

Functional domains of the receptor-associated protein (RAP)

(gp330/low density lipoprotein receptor-related protein/Heymann nephritis/heparin binding/glutathione *S*-transferase)

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ABSTRACT The receptor-associated protein (RAP) specifically associates with gp330 and the low density lipoprotein (LDL) receptor-related protein (LRP), the two newest members of the LDL receptor gene family. Results obtained by ligand blotting, affinity chromatography, and density-gradient sedimentation demonstrate that RAP binds to both receptors with high affinity and that the binding is Ca^{2+} dependent. RAP also binds heparin and is identical to a mouse heparin binding protein (HBP-44) identified in a teratocarcinoma cell line (F9). While biochemical studies have shown that RAP is present on the cell surface and is an effective inhibitor of ligand binding to gp330 and LRP, immunocytochemical findings indicate that RAP is most abundant in the endoplasmic reticulum lumen and may function in receptor folding and/or trafficking. To facilitate the characterization of RAP's function(s) we have mapped its gp330 and heparin binding sites by performing direct binding studies on fusion proteins representing overlapping domains of RAP. gp330 was found to bind to two separate sites on RAP—i.e., between amino acids 85–148 and 178–248. Binding studies with radiolabeled heparin indicate that the heparin binding site is between amino acids 261 and 323, which is consistent with our previously proposed site (residues 287–306) based on the amphipathic nature of the C terminus of RAP. These data demonstrate that the gp330 and heparin binding sites and the Heymann nephritis pathogenic epitope (amino acids 1–86) demonstrated earlier are represented by distinct domains of the RAP polypeptide.

The receptor-associated protein (RAP) is a newly described (1–3) glycoprotein (4) with an apparent molecular mass of 39 kDa in humans (2) and 44 kDa in rats (5). The cDNA for rat RAP, originally called clone 14, was isolated from a rat kidney library (1) during an attempt to identify proteins involved in Heymann nephritis (HN), an experimental autoimmune disease in rats. We have recently identified a pathogenic epitope in RAP (amino acids 1–86) that plays a significant role in formation of subepithelial immune deposits during the initial events of HN (6). Two additional proteins were subsequently cloned and sequenced—i.e., α_2 -macroglobulin RAP (2) and a mouse teratocarcinoma cell (F9) heparin binding protein (HBP-44) (7)—that proved to be the human and mouse homologues of the rat protein. RAPs from all three species have been shown to be immunologically identical (5, 8) and to have extensive sequence homology (1, 2, 9).

Immunoprecipitation studies have shown that RAP specifically associates with gp330 (3, 5, 10) and the low density lipoprotein (LDL) receptor-related protein (LRP) (2, 10)—two large cell-surface receptors that, based on sequence homology (11, 12), are members of the LDL receptor gene family. gp330 is localized to clathrin-coated pits of several epithelia, including the proximal tubule and glomerular epithelia of the kidney (13, 14) and plays a well-defined role in

both the initiation and subsequent progression of HN (5, 15). LRP apparently functions as a multiligand receptor that serves to mediate the hepatic clearance of remnant lipoproteins and plasma proteases complexed with their inhibitors (12, 16, 17). The normal functions of gp330 are currently unknown but are thought to be similar to those of LRP, because by ligand blot analysis gp330 and LRP share similar ligand binding properties (18, 19). However, it remains to be seen whether gp330 and LRP have the same or different physiologic functions. Recent studies have shown that while RAP binding to both gp330 (20, 21) and LRP (10) is Ca^{2+} dependent, it also effectively inhibits ligand binding to both receptors (18, 22, 23). RAP may therefore play a role in modulating ligand binding to these receptors (22). However, the significance of RAP's regulatory role remains unclear since LRP and probably also gp330 (19) are believed to function in the clearance of spent ligands.

Newly synthesized RAP associates with gp330 in the endoplasmic reticulum (ER) (4, 21) and forms a heterodimeric complex that is stable to detergent extraction and high-speed centrifugation (5, 21). RAP has also been shown by immunocytochemistry and/or cell-surface radioiodination to be present on the apical surface of renal proximal tubule cells (1, 5), gingival fibroblasts (2), a rat yolk sac carcinoma cell line (24), and mouse F9 teratocarcinoma cells (8), but by immunocytochemistry it is most abundant in the ER lumen (5, 4), suggesting that it may have an intracellular function—e.g., as a molecular chaperone (2, 14). The fact that RAP has a C-terminal ER retrieval signal (HNEL) is in keeping with its predominant ER localization.

To begin to understand the structural/functional properties of RAP, we have mapped the domains that bind gp330 and heparin by carrying out direct binding studies on immobilized recombinant fusion proteins (fp) representing defined amino acid sequences in RAP. Our data have allowed us to generate a functional map of RAP demonstrating that the HN pathogenic epitope and the gp330 and heparin binding sites are represented by distinct domains.

MATERIALS AND METHODS

Construction of RAP–Glutathione *S*-Transferase (GST) fp. cDNA fragments encoding defined amino acid sequences within RAP were isolated by using either restriction endonuclease digestion or PCR amplification followed by agarose gel electrophoresis and glass powder purification. For PCR, amplification was achieved by using 1.5 units of Pfu (Stratagene), 100 nM primers, 500 ng of RAP cDNA, and 5% dimethyl sulfoxide (DMSO) in a total reaction vol of 100 μ l. Reactions were cycled 30 times with denaturing, annealing, and extension temperatures of 95°C, 40°C, and 75°C, respectively. PCR products were then digested with *Bam*HI and

Abbreviations: RAP, receptor-associated protein; HN, Heymann nephritis; ER, endoplasmic reticulum; LRP, low density lipoprotein receptor-related protein; F-hep, fluoresceinamine-heparin; fp, fusion protein(s); GST, glutathione *S*-transferase; GBM, glomerular basement membrane; BSA, bovine serum albumin.

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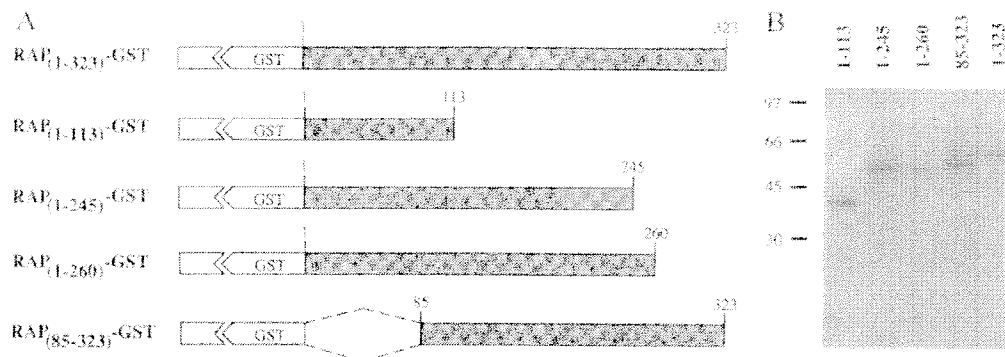


Fig. 1. GST fusion proteins representing defined amino acid sequences within RAP were constructed for use in direct binding studies. (A) RAP cDNA fragments were obtained by using convenient restriction endonuclease sites and used to generate the recombinant fp in the GST prokaryotic expression system. Numbers correspond to amino acids from RAP and do not include the signal sequence. (B) Affinity-purified fp (500 ng) were separated by SDS/10% PAGE. Coomassie staining demonstrates that each fp preparation is >95% pure. Relative molecular masses include 29 kDa for the GST portion of the fp and are as follows: 1-113, 42 kDa; 1-245, 56 kDa; 1-260, 58 kDa; 85-323, 56 kDa; 1-323, 64 kDa.

EcoRI (25). For fragments obtained by restriction endonuclease digestion, the ends were modified to facilitate directional cloning into the *Bam*HI/*Hind*III sites in the prokaryotic expression vector pGEX2T (Pharmacia). Ligation reactions were used to transform *Escherichia coli* strain 71/18 and selected for ampicillin resistance. Bacterial colonies were grown in L broth containing 200 μ g of ampicillin per ml until turbid and then were treated with 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) for 3 hr at 37°C. Culture samples were boiled in reduced Laemmli sample buffer for 5 min and subjected to SDS/PAGE. Colonies expressing the fusion protein construct were stored at -70°C in L broth containing 7% DMSO.

Purification of Recombinant GST fp. RAP-GST fp were purified as described (26) with a few modifications. Cultures were grown overnight in 10 ml of L broth containing 200 μ g of ampicillin per ml, diluted 1:10 into L broth with ampicillin, grown for an additional 1.5 hr, and treated with 0.5 mM IPTG for 3 hr. Cultures were centrifuged at 2500 \times g for 10 min at 4°C, resuspended in 4 ml of 50 mM Tris-HCl, pH 8.0/150 mM NaCl/15% sucrose/50 mM EDTA, and incubated with 1 mg of lysozyme per ml (Sigma) on ice for 1 hr; 4 ml of 1% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride was then added and the mixture was shaken vigorously. The lysate was passed successively through 20- and 27-gauge needles (five times), diluted 1:3 with buffer A [20 mM Hepes, pH 7.6/100 mM KCl/20% (vol/vol) glycerol], and incubated with glutathione-agarose (Sigma) for 16 hr at 4°C. A column was then prepared with the suspension and washed with buffer A until A_{280} achieved baseline. Bound material was eluted with buffer A containing 25 mM reduced glutathione (Sigma), dialyzed exhaustively against PBS, and concentrated with Aquacide II (Calbiochem); purity was determined by SDS/PAGE and/or reverse-phase HPLC. Protein concentration was determined by the BCA assay (Pierce).

Protein Iodinations. gp330 was purified from rat kidney microvilli by affinity chromatography as described (5) and iodinated by the Iodo-Gen (Pierce) method (27). Specific activities for all iodinations were routinely 20–50 \times 10³ cpm per ng of protein.

Solid-Phase Binding Assay. Purified fp (10 μ g/ml) were immobilized onto 96-well high-protein binding plates (Corning) for 16 hr at 4°C. The plates were rinsed two times with buffer B (20 mM Hepes, pH 7.4/80 mM NaCl/2 mM CaCl₂), and nonspecific sites were blocked with buffer B containing 3% bovine serum albumin (BSA) for 1 hr at 23°C. ¹²⁵I-labeled gp330 (¹²⁵I-gp330) was diluted to the indicated concentrations in buffer B with 3% BSA and incubated with the immobilized fp for 3 hr at 23°C, after which the wells were washed three

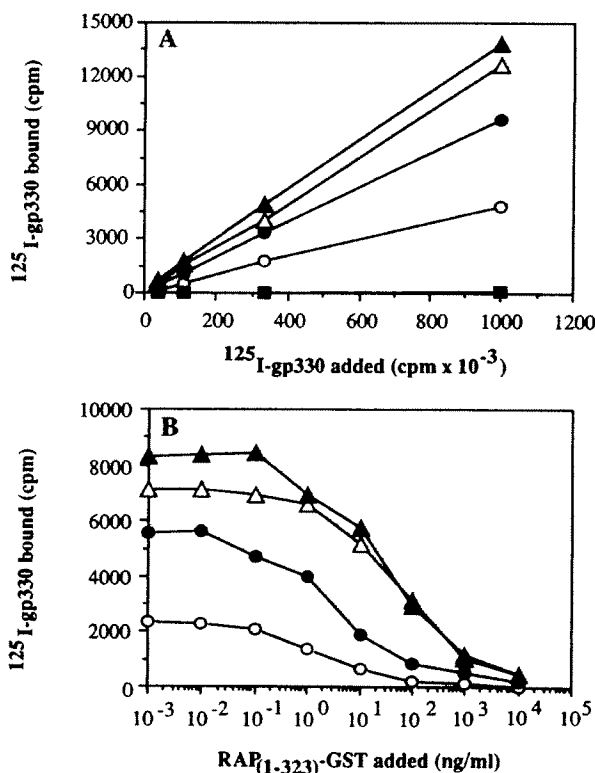


Fig. 2. Direct binding (A) and inhibition (B) studies demonstrate that the main gp330 binding site on RAP is located between amino acids 85 and 245. (A) fp were immobilized on microtiter plates and incubated with radioiodinated gp330 at the indicated concentrations (cpm per well). Bound protein was removed and measured by gamma-counting. gp330 bound to amino acids 85–323 (Δ) and to the complete RAP sequence (1–323) (\blacktriangle) with equivalent affinities, indicating that the receptor binding site on RAP is C terminal to amino acid 85. While the fp representing amino acids 1–245 (\bullet) also bound a significant molar amount of the receptor, it shows a reduced affinity toward gp330. This suggests that the receptor binding site is N terminal to amino acid 245. fp expressing amino acids 1–113 (\circ) bound a small but reproducible amount of gp330. GST alone (\blacksquare) demonstrated no affinity toward gp330. (B) ¹²⁵I-gp330 (5 \times 10³ cpm per well) was incubated with the immobilized fp in the presence of RAP₍₁₋₃₂₃₎ at the indicated concentrations. The binding of ¹²⁵I-gp330 to each of the fp constructs was blocked by competition with full-length RAP₍₁₋₃₂₃₎ in a dose-dependent manner, indicating that the fp interaction with gp330 is highly specific.

times with buffer B. Bound ^{125}I -gp330 was released with boiling 2 M NaOH and measured in a Beckman model 5500B gamma-counter.

Electrophoresis and Ligand Blotting. Proteins were separated by SDS/PAGE (28) and transferred to poly(vinylidene difluoride) Immobilon-P membranes (Millipore) for ligand blotting. Transfer membranes were blocked for 1–16 hr at 4°C with buffer B containing 0.1% Tween-20 (Fisher) and 3% BSA, followed by incubation (3 hr at 23°C) with ^{125}I -gp330. The blots were then washed for 10 min (four times) with buffer B, dried, and exposed to x-ray film at -70°C .

^{125}I -Labeled Fluoresceinamine-Heparin (F-Hep) Overlay. F-Hep was prepared and radioiodinated as described (29) and used for gel overlays according to the procedure of Mehlman and Burgess (30). Briefly, 500 ng of the indicated fp was separated by SDS/PAGE and fixed for 20 min (two changes) with 25% isopropanol/10% acetic acid. The gel was then rinsed with buffer C (50 mM Tris-HCl, pH 7.4/300 mM NaCl) for 20 min (three changes), followed by a 2-hr incubation with buffer C containing 5 mg of BSA per ml at 23°C. The gel was then incubated overnight at 4°C with ^{125}I -F-Hep diluted in buffer C with 5 mg of BSA per ml. After washing with buffer C (four times, 30 min each), the gel was stained with 0.01% Coomassie blue R (15 min), dried, and exposed to X-Omat AR film (Kodak).

RESULTS

Construction of fp Representing Subdomains of RAP. To identify the domain(s) within the RAP polypeptide that binds to gp330, we constructed recombinant fp representing de-

fined amino acid sequences—i.e., 1–113, 1–245, 1–260, and 85–323—from RAP (Fig. 1A). The fp were then used in a direct solid-phase binding assay. fp were purified from bacterial lysates by affinity chromatography and separated by SDS/PAGE to verify their size and purity. As shown in Fig. 1B, the fp demonstrated >95% purity by Coomassie blue staining and were of the appropriate molecular masses (including 29 kDa for the GST portion of the fp). HPLC separation by reverse-phase chromatography further substantiated the purity of the fp (data not shown).

The gp330 Binding Site on RAP Is Between Amino Acids 85 and 245. To establish the binding potential and specificity of each fp for gp330, we performed direct binding studies. When the purified fp were immobilized onto protein binding plates and incubated with radioiodinated gp330, RAP_(1–323) and RAP_(85–323) both bound similar molar amounts of gp330 and had equal binding affinities (Fig. 2A). RAP_(1–245) bound gp330 with a lower affinity compared to the full-length RAP_(1–323). This reduced affinity is most likely due to modifications in the tertiary structure of RAP_(1–245) resulting from the deletion of C-terminal amino acids in the fp construct (i.e., amino acids 246–323). However, this construct bound similar molar amounts of gp330 as the full-length RAP_(1–323). RAP_(1–113) demonstrated a slight affinity for gp330, suggesting that some sequence information is present within this construct that contributes to gp330 binding.

The Binding of gp330 to the RAP fp Constructs Is Highly Specific. To assess the binding specificity between gp330 and the fp we performed competition studies with full-length RAP_(1–323). Radiolabeled gp330 and varying amounts of RAP_(1–323) were coincubated with the immobilized fp and the

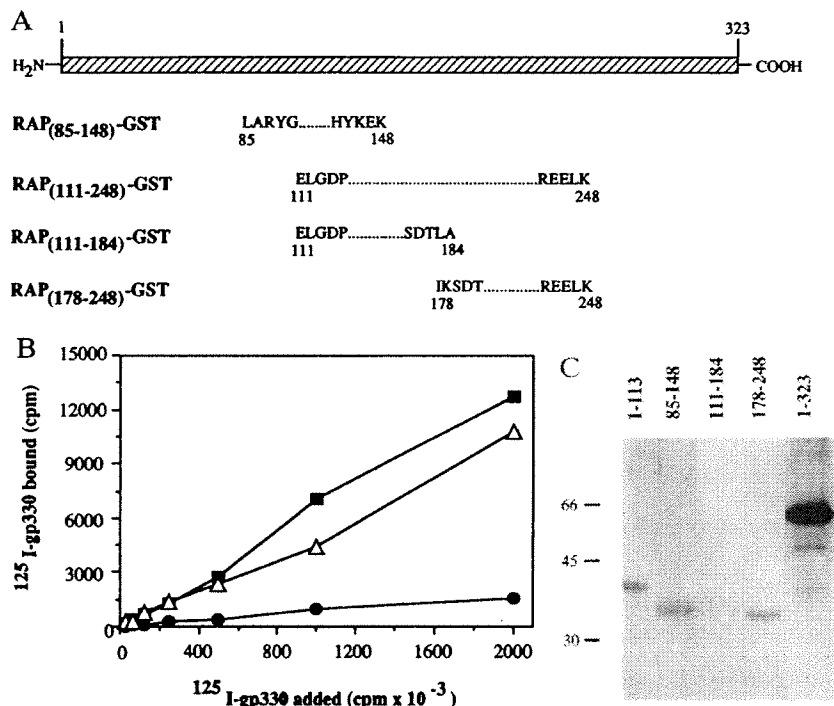


FIG. 3. RAP includes two binding sites for gp330. (A) PCR was used to generate cDNA fragments representing the amino acids shown, which were subsequently cloned into the pGEX2T prokaryotic expression vector. Amino acid numbering is based on the first residue following cleavage of the signal sequence. (B) ^{125}I -gp330 was incubated at the indicated concentrations (cpm per well) with immobilized fp representing amino acids 111–248 (■), 111–184 (●), and 178–248 (Δ). gp330 bound to residues 111–248 and its C-terminal half, residues 178–248. However, no specific binding was detected to amino acids 111–184, indicating that a gp330 binding site resides between amino acids 178 and 248. (C) Purified fp expressing the RAP amino acid sequences shown were separated by SDS/12% PAGE and analyzed by ligand blotting with ^{125}I gp-330 (2×10^5 cpm/ml). By this method, radiolabeled gp330 bound to amino acids 1–113 and 178–248, confirming the results obtained from the solid-phase binding assay. In addition, residues 85–148 also bound gp330, thereby identifying a second receptor binding site on RAP. No binding of gp330 to amino acids 111–184 was detected.

amount of bound gp330 was determined. As shown in Fig. 2B, RAP₍₁₋₃₂₃₎ effectively competed for the binding of gp330 to all of the fp constructs in a dose-dependent manner. These results indicate that the binding of gp330 to the fp is highly specific and the fp have a conformation that is representative of the native binding site.

gp330 Binds to Two Sites on RAP Between Amino Acids 85 and 245. To further narrow the gp330 binding site(s) on RAP and investigate the binding affinity demonstrated by RAP₍₁₋₁₁₃₎, we used PCR technology to generate fp constructs of smaller defined amino acid lengths. Fig. 3A shows schematically the RAP polypeptide sequences whose corresponding cDNAs were amplified and used to construct fp expressing amino acids 111–248, 111–184, 178–248, and 85–148. The purified RAP constructs were immobilized and incubated with radiolabeled gp330. gp330 bound to both RAP₍₁₁₁₋₂₄₈₎ and its C-terminal half, RAP₍₁₇₈₋₂₄₈₎ (Fig. 3B). However, little or no binding was detected to RAP₍₁₁₁₋₁₈₄₎, indicating that a binding site on RAP for gp330 is located between amino acids 178 and 248. To determine whether RAP₍₁₋₁₁₃₎ fp expressed a second receptor binding site in addition to that between residues 178 and 248, we performed a ligand blot analysis by incubating ¹²⁵I-gp330 with the fp constructs that had been electroblotted to poly(vinylidene difluoride) transfer membrane. Both RAP₍₁₋₁₁₃₎ and RAP₍₈₅₋₁₄₈₎ bound radiolabeled gp330 (Fig. 3C), confirming that sequence information within amino acids 1–113 contributes to gp330 binding and that an additional gp330 binding site on RAP is represented by amino acids 85–148. Moreover, by ligand blotting radioiodinated gp330 bound to RAP₍₁₇₈₋₂₄₈₎ but not RAP₍₁₁₁₋₁₈₄₎, which verifies the results obtained in the solid-phase assay.

The Heparin Binding Site in RAP Is Between Amino Acids 261 and 323. Previous studies have shown that RAP can be purified by heparin affinity chromatography (7) and that RAP, but not gp330, directly binds heparin by ligand blotting (24). Based on structural homologies with previously characterized heparin binding motifs (31), we proposed that the heparin binding site in RAP is located between amino acids 287 and 306 (24). To test this hypothesis, we performed heparin overlays (30) on the electrophoretically separated fp in order to identify the location of the glycosaminoglycan

binding site. As shown in Fig. 4A, radiolabeled heparin bound to RAP₍₈₅₋₃₂₃₎ and RAP₍₁₋₃₂₃₎, but not RAP₍₁₋₁₁₃₎ or RAP₍₁₋₂₆₀₎. These results clearly indicate that the heparin binding site in RAP is C terminal to amino acid 260. Furthermore, a coin-cubation of unlabeled heparin (1 mg/ml) completely inhibited the binding of radiolabeled heparin to RAP₍₈₅₋₃₂₃₎ and RAP₍₁₋₃₂₃₎, while chondroitin sulfate (1 mg/ml) was ineffective (Fig. 4B). This confirms that the glycosaminoglycan binding property is specific for heparin and is not due to nonspecific charge interactions with sulfate moieties.

DISCUSSION

In this report, we have generated recombinant fusion proteins representing defined amino acid sequences within RAP in order to identify its gp330 and heparin binding sites. By direct binding studies, we have shown that there are two primary gp330 binding sites within RAP—one between amino acids 85 and 148 and the other between amino acids 178 and 248. Moreover, each site is capable of binding gp330 independently. Whether the interaction of these sites with gp330 is competitive or cooperative remains to be determined. Amino acids 215–248 within the gp330 binding site include a number of aspartic and glutamic acid residues as well as a putative N-linked glycosylation site. This results in a domain with a high negative charge, which may contribute to the Ca²⁺-dependent binding of RAP to gp330 (20, 24). Also, immediately preceding the N-terminal gp330 binding site (amino acids 85–148) are a series of leucine residues separated by a 3- to 5-residue spacing, which may constitute a leucine zipper motif. Leucine zippers were originally identified in DNA binding proteins and have been shown to promote the formation of homodimers (32). While the functional contributions of this motif within RAP are currently unknown, dimerization of RAP in the ER may facilitate the oligomerization of the gp330/RAP heterodimeric complex described previously (5, 21).

Immunizing rats with a renal brush border preparation (active HN), which represents an enriched source of gp330/RAP, leads to formation of glomerular subepithelial immune deposits (33). Although gp330 is a key autoantigen involved in the pathogenesis of HN (14), we have also identified an epitope within RAP that can induce development of immune aggregates (6). In contrast, rats immunized with liver membranes containing LRP/RAP failed to develop any renal abnormalities (33). Based on these results, we have recently proposed three models (5) describing how this epitope in RAP may initiate HN. Since this autoimmune disorder can be induced in rats by administering gp330/RAP but not LRP/RAP, one model suggests that the pathogenic epitope is exposed when RAP is associated with gp330 but is cryptic when bound to LRP. The influence of gp330 or LRP on the conformational state of RAP could affect epitope availability and may explain the differences between kidney and liver membranes in the induction of active HN. This hypothesis is further supported by a recent study in which several domains of RAP were shown to inhibit the binding of tissue-type plasminogen activator and α_2 -macroglobulin to LRP (34). Two of these inhibitory domains correspond to the gp330 binding sequences in RAP that we report here, but two additional regions, encompassing residues 18–24 and 311–319, were identified that are also capable of blocking ligand binding to LRP. The presence of two additional LRP binding sites on RAP suggests that there may be differences in the conformation of RAP when associated with LRP vs. that when it is associated with gp330. With the sequences now known that mediate receptor binding to RAP, it should be possible to determine whether these conformational differences do exist and whether they affect epitope presentation in HN.

The immune deposits that form in HN between the glomerular basement membrane (GBM) and the base of the

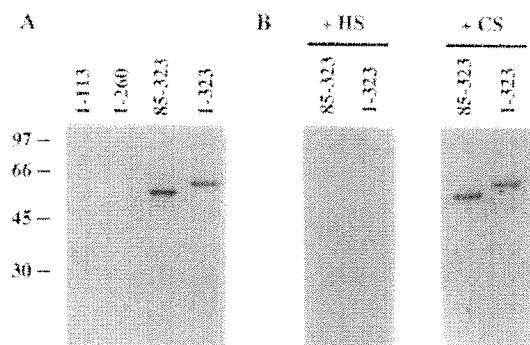


FIG. 4. The heparin binding site on RAP is located between amino acids 261 and 323. (A) Purified fp were separated by SDS/10% PAGE and subsequently incubated with ¹²⁵I-F-hep (2×10^5 cpm/ml). fp representing the complete RAP sequence (residues 1–323) and amino acids 85–323 bound the radiolabeled glycosaminoglycan. However, residues 1–113 and 1–260 did not bind ¹²⁵I-F-hep, indicating that the binding site for heparin on RAP is between amino acids 261 and 323. (B) To determine the specificity of heparin binding, fp were incubated with ¹²⁵I-F-hep in the presence of either excess unlabeled heparin sulfate (HS) or chondroitin sulfate (CS). The binding of ¹²⁵I-F-hep to the fp was effectively blocked by competition with heparin but not chondroitin sulfate, indicating that heparin binding to amino acids 261–323 is specific and not due to nonspecific charge interactions with the sulfate moieties. Numbers on left are kDa.

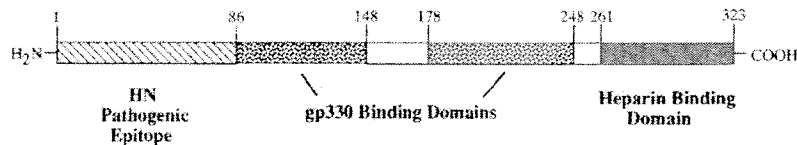


FIG. 5. Domain map of RAP based on results of direct binding of gp330 and heparin to recombinant fp representing defined amino acid sequences in RAP. We have identified two primary binding sites on RAP for gp330 (amino acids 85–148 and 178–248). In addition, we have also identified a binding site for heparin (amino acids 261–323). Based on structural homologies with other well-characterized heparin binding proteins, RAP contains a putative heparin binding motif (amino acids 287–306) within the heparin binding domain identified here. A pathogenic epitope capable of inducing HN was previously identified between amino acids 1 and 86 (6) and was shown to play a significant role in development of subepithelial immune deposits.

epithelial foot processes eventually become crosslinked to the GBM (15), which contains abundant heparan sulfate proteoglycans (HSPGs) (35, 36). These antibody–antigen aggregates ultimately disrupt the filtration properties of the GBM, leading to proteinuria and severe glomerular injury. Our earlier studies on defining the structural/functional role of heparan sulfate on the gp330/RAP revealed that RAP, but not gp330, specifically binds heparin (24). Although heparin is able to inhibit the binding of RAP to gp330 (10), it does not cause dissociation of the gp330/RAP heterodimer (24). These results suggest that HSPGs in the GBM may play a role in the binding of antibody–antigen complexes to the GBM through gp330/RAP. We have previously pointed out that the C terminus of RAP includes a region (amino acids 287–306) of high positive charge density that could interact with the highly sulfated heparin/heparan moiety. Using a radiolabeled heparin overlay procedure (30), we have directly demonstrated in this study that the heparin binding site on RAP is between amino acids 261 and 323. These results show that the amino acid sequences in RAP that bind gp330 and heparin are noncontiguous and are consistent with the fact that the glycosaminoglycan site is physiologically exposed when RAP is bound to gp330 (24).

Collectively, the data presented in this and an earlier (6) report have allowed us to construct a map of the functional domains of RAP (Fig. 5). The key finding is that the gp330 binding sites, the heparin binding site, and the HN pathogenic epitope are found in distinct domains of RAP. This information will facilitate future studies aimed at characterizing the inhibitory properties of RAP toward receptor–ligand interactions and at defining the potential role of RAP in the trafficking and processing of gp330 en route to the cell surface.

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